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Experimental Hybridization of the Green Treefrog *Hyla cinerea* Schneider (Hylidae)

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Plates I & II

EXTERNAL fertilization in most frogs and toads permits the investigator to attempt fertilization between selected anuran gametes and to observe subsequent stages of development. This relatively easy access to the gametes of most anurans has been used to advantage in studies of amphibian genetics (Pyburn, 1961), embryology (Cusimano, Fagone & Reverberi, 1962), cytology (Moore, 1961), evolution (A. P. Blair, 1941; Mecham, 1961). Numerous other pertinent studies could be cited. The objective in making the series of crosses described in this account was to determine if the evidence obtained from experimental hybridization supports judgements of relationship between these hylid species that have been made in the absence of such information.

Gross morphology has long been the major, if not single, source of evidence underlying most judgements of anuran relationships but with the availability and the refinement of techniques which permit the analysis and comparison of amphibian breeding calls (W. F. Blair, 1958c), seroprotein patterns (Bertini & Cei, 1961), chromosomes (Sanders & Cross, 1964), and others, such sources of information can no longer be ignored when the most convincing statement of anuran species relationships is desired. One additional source of information is experimental hybridization and if it be assumed that closely related species are more likely to form viable hybrids between them when experimentally crossed than they are when crossed to more distantly related species, then it seems reasonable to attempt experimental hybridization whenever possible and to make available this evidence so that it might be considered in evaluating relationships between the species crossed. Information from experimental hybridization has proved useful in phylogenetic studies of the genus *Bufo* (W. F. Blair, 1963) but a comparable quantity of data has been lacking for members of the genus *Hyla*.

Materials and Methods

All eggs used in these crosses were produced by two female *Hyla cinerea*. These females are distinguished by letter in Tables 1 and 2. Fertilization of *H. cinerea* eggs was attempted using sperm suspensions from *H. cinerea, H. crucifer, H. versicolor, Acris crepitans* and *Pseudacris clarki*.

The method of experimental hybridization is similar to those described by Pyburn & Kennedy (1960). In brief, eggs of ovulating females were introduced into separate sperm suspensions prepared from the testes of individual males. Embryos were maintained in enamel pans containing water. Larvae were transferred to 5-gallon aquaria. At metamorphosis the young frogs were placed in laboratory cages containing a basal layer of damp soil. They were fed laboratory-raised *Drosophila, Tenebrio* larvae and small insects caught by sweeping with insect nets. All frogs used in this study, except *Hyla crucifer*, were collected by me.

Results

Results of crosses with two female *H. cinerea* (A, B) are summarized in Tables 1, 2. The latest stage of development attained by the offspring of the following combinations has been listed by Pyburn & Kennedy (1961).

♀ *Hyla cinerea* (A) × ♂ *Hyla versicolor*. Most eggs cleaved but failed in gastrulation. Twenty-six larvae hatched beginning on the third day

TABLE 1. Crosses between a female *Hyla cinerea* (A) from Fort Bend County, Texas, and males of the species in the left hand column. Localities from which the males were collected are listed beneath each. Percentages of hatch and metamorphosis are based on number of cleaved eggs.

Male	Eggs	Cleavage Number (%)	Hatch %	Metamorphosis %	Development
Hyla versicolor Fort Bend Co., Texas	485	462 (95.7)	5.6	1.5	Adult
Acris crepitans San Patricio Co., Texas	156	130 (83.3)	49.2	0.0	Tadpole
Hyla cinerea (control) Fort Bend Co., Texas	157	157 (100.0)	96.8	35.1	Adult

after fertilization. Two hybrids lacked the left eye. Three hybrids could not close the mouth. The lower jaw was permanently deflected so that the tongue was always visible (Plate I). The remaining, apparently normal, hybrid was inadvertently crushed by the lid of the cage.

Shortly after metamorphosis, the young frogs changed from a pale green to gray with dark dorsal markings similar to the male parent. The white subocular spot characteristic of *H. versicolor* was evident in all frogs obtained from this cross. All of the hybrids except two individuals with deformed mouths died within 18 days after metamorphosis. One individual died 10 months and 2 days after fertilization and had a snout-vent measurement of 38 mm. after preservation. This frog was always the smaller of the two surviving hybrids and was usually pale green (Plate I B). The other individual (Plate I A) has faint sexual coloration of the throat skin. This hybrid has not called in the laboratory but sometimes chirps when picked up. Even though about the anterior one-half of the lower jaw was permanently deflected in both of these hybrids, they could eat mealworms and small grasshopper nymphs. They could not catch small leafhoppers and small flying insects. Internal structures in these two hybrids were poorly preserved. The gonads could not be located upon dissection and may have been lacking.

Two crosses involving a ♂ *H. cinerea* and ♀ *H. versicolor* have been reported. Tadpoles were obtained from one such combination, but they did not transform and all of the tadpoles died within a few days after hatching (Pyburn & Kennedy, 1960). One young frog was obtained from this combination by Littlejohn (1961) but this frog died about two weeks after transformation. Both of these crosses of ♂ *H. cinerea* × ♀ *H. versicolor* resulted in a lower percentage of development than the reciprocal cross.

♀ *Hyla cinerea* (A) × ♂ *Acris crepitans.* All of the surviving embryos were in gastrula one day after fertilization but gross abnormalities in gastrulation hindered development. Many embryos could not incorporate the abnormally large yolk plugs and did not show subsequent development. Others had greatly distended bellies. The first individual hatched five days after fertilization.

No frogs have been obtained from crosses of *Acris crepitans* with any U. S. member of the genus *Hyla.*

♀ *Hyla cinerea* (A) × ♂ *Hyla cinerea* (Control). All of the 157 eggs cleaved and no abnormalities were observed in the early developmental stages. One hundred fifty-two young tadpoles were obtained of which 55 metamorphosed. Mortality in the tadpole stage was probably due to space limitations. There were no external abnormalities among the young frogs. All were pale green with a distinct lateral white stripe that was variable in width and length. In some individuals, the white stripe had

TABLE 2. Crosses between a female *Hyla cinerea* (B) from Fort Bend County, Texas, and males of the species in the left hand column. Localities from which the males were collected are listed beneath each. Percentages of hatch and metamorphosis are based on number of cleaved eggs.

Male	Eggs	Cleavage Number (%)	Hatch %	Metamorphosis %	Development
Hyla crucifer Wyoming Co., Penn.	197	158 (80.0)	2.6	—	Tadpole
Pseudacris clarki Harris Co., Texas	260	156 (60.0)	4.5	—	Tadpole

a narrow black border (Plate II A). The golden chromatophores evident as irregular dots on the dorsum of adult *H. cinerea* were not evident in the frogs immediately after metamorphosis but appeared as the frogs increased in size.

An F_1 male of 43 mm. snout-vent length called 58 days after fertilization. The air temperature in the laboratory was 24.4 C. This was the first call heard from these frogs, although various males called at irregular intervals thereafter. The call sounded like those of typical wild male *cinerea*. Twenty-four frogs from the control cross were preserved. The range in snout-vent length is 30-48 mm., with a mean of 36 mm. There were 18 males and 6 females. The observed sex ratio differs significantly from a theoretical 1:1 ratio beyond the 5% level of probability ($\chi^2 = 5.04$). If the expected 1:1 sex ratio is correct, then either fewer females than males survived or fewer females than males were produced from this cross. It is also possible that the 24 frogs do not represent a random sample.

Microscopic sections of the ovaries of one female showed that normal oogenesis was in progress but that no mature eggs had been formed. This female was preserved 128 days after fertilization and her snout-vent length was 35 mm. Histological preparations were not necessary to determine the stage of oogenesis in three females because their ovaries contained large masses of well-developed eggs. Snout-vent lengths of these females were 40, 42 and 45 mm. Their oviducts were evident upon dissection as slightly convoluted tubes. All three females were preserved and measured 500 days after fertilization. The ovaries of two other females, 33 and 37 mm. snout-vent lengths, did not contain eggs that were visible upon dissection. Poor preservation made histological study of the ovaries not feasible. There is no evidence to indicate that normal oogenesis was not occurring in each of these females.

Microscopic sections of the testes of 17 males were examined. The testes of one frog were not examined histologically because of poor preservation. Spermatozoa were present in the testes of all but one of 17 control males. The mean snout-vent length of these individuals is 41.4 mm. (30-48 mm. range). The frog whose testes did not possess spermatozoa has a snout-vent length of 39 mm., which is well within the range of sexual maturity indicated by the control males. The left testis of the sterile frog was the smallest of the group measured, having a length of 3.0 mm. and a width of 1.0 mm. Measurements of snout-vent length and testes length and width were made on 14 apparently fertile F_1 controls. The mean snout-vent length is 41.5 mm. (30-48 mm. range). The mean length of the left testis of these 14 males is 4.5 mm. (3.3-6.0 mm. range); the mean width is 2.0 mm. (1.7-2.6 mm. range). The testes of two additional frogs were not measured but contained spermatozoa. The only abnormality noted upon dissection of the controls was that one frog had a greatly distended urinary bladder which contained a copious white mucus.

♀ *Hyla cinerea* (B) × ♂ *Hyla crucifer.* Eighty percent. of the 197 eggs cleaved and developed to blastula without major abnormalities, but only 40% attained gastrula (Table 2). Difficulties in gastrulation resulted in most of the mortality, and only 10% developed into neurula. Four larvae hatched about three days after fertilization but one died shortly after hatching. Two of the young tadpoles died within 10 days after fertilization. The remaining tadpole was notably abnormal. The tail was deflected and the body was larger than normal in proportion to the tail. These abnormalities did not seem to interfere with feeding. Front limbs had not appeared when this individual died 72 days after fertilization. The labial tooth formula is $\frac{2}{2.}$ The upper horny beak is denticulate. A lower beak is not present.

♀ *Hyla cinerea* (B) × ♂ *Pseudacris clarki.* One hundred thirty embryos developed into gastrulae, but only 19 embryos attained the stage of tail bud. Seven larvae hatched beginning on the third day after fertilization. Although the young tadpoles were very active and did not appear abnormal, all but three died within five days after hatching. The last surviving individual died seven days after hatching. One larva hatched from the reciprocal combination, ♀ *Pseudacris clarki* × ♂ *Hyla cinerea;* all embryos were twisted and the one that hatched died soon afterward (Littlejohn, 1961).

Discussion

All of the crosses were attempted between members of the family Hylidae. Thus the species crossed share some common morphology at least at the familial level. Two of the crosses which failed to produce F_1 frogs were intergeneric combinations of *Hyla cinerea* with *Acris crepitans* and *Pseudacris clarki.* The morphology of *A. crepitans* and *P. clarki* would not appear to support an argument for a close relationship to the larger and arboreal *Hyla cinerea* (Wright & Wright, 1949). The breeding calls of these species have been analyzed by W. F. Blair (1958a, b, c) and do not suggest a close relationship. It can tentatively be concluded from the single cross of ♀ *H. cinerea* × ♂ *Pseudacris clarki* that this combination shows reduced survival and that the

tadpole is not capable of metamorphosing. Genetic incompatibility is expressed in the stages preceding metamorphosis and largely in the pretadpole stage. I am fully cognizant that no control cross was attempted for this experimental cross and that the results of single crosses must be interpreted with caution. The tadpoles obtained from the ♀ *Hyla cinerea* × ♂ *Acris crepitans* failed to metamorphose. The occurrence of a sterile adult *H. cinerea* male in the F_1 controls (Table 1) points out the advisability of checking suspensions in which the testes of wild-caught males have been macerated for the presence of sperm capable of fertilization.

Another combination that did not produce adults is that of ♀ *H. cinerea* × ♂ *H. crucifer*. *Hyla crucifer* does not appear to be closely related to *H. cinerea* on the basis of morphological evidence. According to Lynch (1962) the presence of the ilial shaft ridge in *Hyla crucifer*, *Acris crepitans* and *Acris gryllus* may indicate that *H. crucifer* is more closely related to *Acris* than *Hyla*. The ilial shaft ridge is not present in the species of *Hyla*, which included *H. cinerea*, examined by Lynch. The number of maxillary teeth is considerably greater in *Hyla cinerea* than in *Acris crepitans*, *Pseudacris clarki* and *Hyla crucifer* (Goin, 1958). The call of *H. crucifer* does not fit closely to any U. S. *Hyla* but one type of *H. crucifer* call shows resemblances to calls of *Pseudacris streckeri* and *P. ornata* (W. F. Blair, 1958a). Hybrid inviability in the combination of *Hyla cinerea* × *Hyla crucifer* was expressed in the stages preceding metamorphosis, but this conclusion is uncertain because no control cross was made. However, the available information from this cross and other sources does not indicate a close relationship of *H. crucifer* with *H. cinerea*.

Differences in chromosome number could contribute to hybrid inviability and the chromosomes of several of the frogs hybridized have been studied. According to Bushnell, Bushnell & Parker (1939), 12 is the haploid chromosome number of *H. versicolor* and *H. cinerea*. The chromosomes of these species are similar in that the haploid complement consists of seven large and five smaller chromosomes. Adults were produced in the hybrid combination of *H. versicolor* with female *H. cinerea*. Difference in chromosome number is a potential source of hybrid inviability in the combination with *Acris crepitans* in which the haploid number is 11 (Bushnell, Bushnell & Parker, 1939), and with *H. crucifer* in which the haploid number is probably 13 (Witschi, 1933).

Results of the combination between *Hyla cinerea* and *Hyla versicolor* suggest a greater degree of genetic compatibility between these two species than was suspected solely on the basis of their external morphology. The percentage of hatch and metamorphosis from this combination is considerably lower than in the controls. Even though adults were obtained, development from this cross was limited and a reduction in interfertility is indicated. The calls of the two species show sufficient divergence to place them in different species groups. This and other evidence suggests that the closer affinities of *Hyla cinerea* lie within the *cinerea* species group (W. F. Blair, 1958a). Natural hybrids between *Hyla cinerea* and one member of the *cinerea* species group, *Hyla gratiosa*, have been reported by Bogert (1960) and Mecham (1960). Results of the crosses presented here do not suggest a close relationship between *Hyla cinerea* and *Hyla crucifer*, *Hyla versicolor*, *Pseudacris clarki* or *Acris crepitans*. Additional crosses are needed before the full potential of genetic compatibility between these species can be realized.

Statements of the complexities in the use of experimental hybridization (Moore, 1955, 1959; Clark Hubbs, 1963), can be used to avoid inaccuracy in the interpretation of hybridization results. If anuran postmating isolating mechanisms cannot be reinforced by selection (Mecham, 1961), then this complication may not be as pertinent in influencing the degree of crossability as has been thought. Even with other acknowledged complications, the data from experimental hybridization are useful in the determination of anuran relationships (W. F. Blair, 1962) and justify consideration along with the more conventional sources of evidence.

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Summary

Series of experimental crosses were made to determine if the evidence from experimental hybridization supports current judgements of relationships between the species crossed. Four hybrid and one control combination were made using eggs from two *Hyla cinerea*. If the latest stage of development be used as a crude expres-

sion of genetic compatibility, then the results of hybridization tests generally support judgements of relationship between the species crossed but the evidence presented here must be interpreted with caution, for problems inherent in experimental hybridization, such as gynogenesis and others (see Clark Hubbs, 1963), were not clearly excluded.

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EXPLANATION OF THE PLATES

Plate I

Fig. 1. **A.** F_1 ♂ (♀ *Hyla cinerea* (A) × ♂ *Hyla versicolor).* **B.** Same cross, different individual. Neither hybrid could close its mouth. The white subocular spot characteristic of *versicolor* is shown.

Plate II

Fig. 2. F_1 (♀ *Hyla cinerea* (A) × ♂ *Hyla cinerea*) controls. Variability of the lateral stripe is shown. **A.** 47 mm. snout-vent frog was very dark green when photographed. **B.** 45 mm. snout-vent frog was very light green when photographed.